IN THE MATTER OF two
German Patent Applications
in the name of
Gesellschaft fuer Biotechnologische Forschung mbH (GBF)
filed under
~~196 47 580.5 and~~ 197 07 506.1
and
IN THE MATTER OF an Application
for Patent in Singapore

I, Dr. Hans D. Boeters, translator and patent attorney of BOETERS & BAUER, Bereiteranger 15, D-81541 Muenchen, Germany, do solemnly and sincerely declare that I am conversant with the English and German languages and am a competent translator thereof, and that the following is, to the best of my knowledge and belief, a true and correct translation of the patent applications filed under ~~196 47 580.5 and~~ 197 07 506.1

by Gesellschaft fuer Biotechnologische Forschung mbH (GBF)

before the German Patent Office on December 18, 1996 and February 2, 1997

for "Epothilon E and F" ~~and "Epothilons C and D, preparations and compositions"~~

and the Official Certificates attached thereto.

Date: June 9, 1999

(Dr. Boeters)

FEDERAL REPUBLIC OF GERMANY

CERTIFICATE

Gesellschaft für Biotechnologische Forschung mbH (GBF) of Braunschweig/Germany filed at the German Patent Office on 25th February, 1997 a Patent Application entitled

"Epothilon E and F".

The attached documents are a correct and accurate reproduction of the original supporting documents of this Patent Application.

The Application has provisionally been given in the German Patent Office the symbols C 07 D, C 12 P and A 61 K of the International Patent Classification.

Seal of the German
Patent Office

Munich, 19th December 1997
The President of the German
Patent Office
By order

(signature)

File reference: 197 07 506.1

(Joost)

Epothilon E and F

Production strain:

The production strain *Sorangium cellulosum* So ce90 was isolated in July 1985 at GBF from a soil sample obtained from the banks of the Zambesi and deposited with the Deutsche Sammlung für Mikroorganismen [German Collection of Microorganisms] under No. DSM 6773 on 28.10.91.

Details of the producer and the culture conditions are described in:
Höfle, G.; N. Bedorf, K. Gerth & H. Reichenbach: Epothilons, processes for their preparation and compositions containing them. DE 41 38042 A1, laid open to inspection on 27th May 1993.

Formation of epothilons E and F during fermentation:

Typical fermentation proceeds as follows: a 100 litre bioreactor is charged with 60 litres of medium (0.8% starch; 0.2% glucose; 0.2% soya flour; 0.2% yeast extract; 0.1% $CaCl_2 \times 2\ H_2O$; 0.1% $MgSO_4 \times 7\ H_2O$; 8 mg/litre of Fe-EDTA; pH 7.4). 2% Adsorber resin (XAD-16, Rohm und Haas) is also added. The medium is sterilised by autoclaving (2 hours, 120°C). Inoculation is effected with 10 litres of a pre-culture that has been cultured in a shaking flask (160 rpm, 30°C) in the same medium (additionally 50 mM HEPES buffer, pH 7.4). Fermentation is carried out at 32°C at a stirring speed of 500 rpm and aeration of 0.2 Nl per m^3 per hour; the pH value is maintained at 7.4 by the addition of KOH. Fermentation lasts from 7 to 10 days. The epothilons formed are bound continuously to the adsorber resin during fermentation. After the culture broth has been separated off (e.g. by sieving using a process filter), the resin is washed with 3 bed volumes of water and eluted with 4 bed volumes of methanol. The eluate is concentrated to dryness and taken up in 700 ml of methanol.

HPLC analysis of the XAD eluate:

The eluate is in a concentration of 100:1 compared with the starting volume of the reactor (70 litres). The analysis is carried out using a 1090 HPLC apparatus made by Hewlett Packard. A microbore column (125/2 Nucleosil 120-5 C_{18}) made by Machery-Nagel (Düren) is used to separate the contents. Elution is carried out using a water/acetonitrile gradient of initially 75:25 up to 50:50 after 5.5 minutes. That ratio is maintained up to the 7th minute and is then increased to 100% acetonitrile up to the 10th minute. Measurement is made at a wavelength of 250 nm and a band width of 4 nm. The diode array spectra are measured in a wavelength range of from 200 to 400 nm. Two new substances having a R_t of 5.29 and 5.91, respectively, occur in the XAD eluate, the absorption spectra of which are identical to those of epothilons A and B, respectively (Fig. 1: E corresponds to A, F corresponds to B). Only traces of those substances are formed under the given fermentation conditions.

Biotransformation of epothilon A and B into epothilon E and F:

A 500 ml 4-day-old So ce90 culture that is maintained with adsorber resin is used for the specific biotransformation. 250 ml thereof are transferred to a sterile 1 litre Erlenmeyer flask, leaving behind the XAD. A methanolic solution of a mixture of a total of 50 mg of epothilon A + B is then added and the flask is incubated on a shaking cabinet for 2 days at 30°C and 200 rpm. The formation of epothilons E and F is analysed directly from 10 µl of the centrifuged culture residue (Fig. 2). Conversion takes place only in the presence of the cells and depends upon the cell density used and upon time. Kinetics of the conversion are shown for epothilon A in Fig. 3.

Isolation of epothilon E and F

To isolate epothilon E and F, 3 shaking-flask batches from the biotransformation (see above) are combined and shaken for 1 hour with 20 ml of XAD-16. The XAD is obtained by sieving and is eluted with 200 ml of methanol. The eluate is concentrated by evaporation *in vacuo* to yield 1.7 g of crude extract which is partitioned between 30 ml of ethyl acetate and 100 ml of water. From the ethyl acetate phase there are

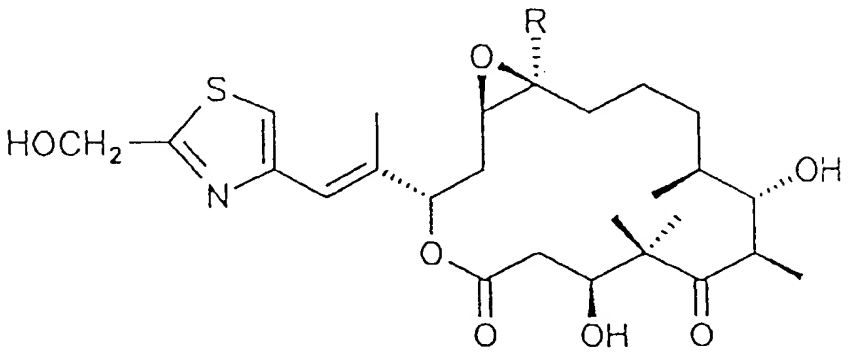
obtained by concentration by evaporation *in vacuo* 330 mg of an oily residue which is chromatographed in 5 runs over a 250 x 20 mm RP-18 column (eluant: methanol/-water 58:42, detection 254 nm.).

Yield: epothilon E 50 mg
F 10 mg

Biological activity of epothilon E:

In cell cultures the concentration that reduces growth by 50% (IC_{50}) was determined and compared with the values for epothilon A.

Cell line	IC_{50} (ng/ml)	
	Epothilon E	Epothilon A
HeLa, KB-3.1 (human)	5	1
murine fibroblasts, L929	20	4



Epothilon E R = H

Epothilon F R = CH_3

EPOTHILON E

$C_{26}H_{39}NO_7S$ [509]
ESI-MS: (positive ions): 510.3 for $[M+H]^+$

TLC: R_f = 0.58
TLC aluminium foil 60 F 254 Merck. Eluant: dichloromethane/methanol = 9:1
Detection: UV extinction at 254 nm. Spraying with vanillin/sulphuric acid reagent, blue-grey coloration on heating to 120°C.

HPLC: R_t = 5.0 min
Column: Nucleosil 100 C-18 7 µm, 250 x 4 mm
Eluant: methanol/water = 60:40
Flow rate: 1.2 ml/min
Detection: diode array

^{1}H-NMR (300 MHz, $CDCl_3$): δ = 2.38 (2-H_a), 2.51 (2-H_b), 4.17 (3-H), 3.19 (6-H), 3.74 (7-H), 1.30-1.70 (8-H, 9-H_2, 10-H_2, 11-H_2), 2.89 (12-H), 3.00 (13-H), 1.88 (14-H_a), 2.07 (14-H_b), 5.40 (15-H), 6.57 (17-H), 7.08 (19-H), 4.85 (21-H_2), 1.05 (22-H_3), 1.32 (23-H_3), 1.17 (24-H_3), 0.97 (25-H_3), 2.04 (27-H_3)

EPOTHILON F

$C_{27}H_{41}NO_7S$ [523]
ESI-MS: (positive ions): 524.5 for $[M+H]^+$

TLC: R_f = 0.58
TLC aluminium foil 60 F 254 Merck. Eluant: dichloromethane/methanol = 9:1
Detection: UV extinction at 254 nm. Spraying with vanillin/sulphuric acid reagent, blue-grey coloration on heating to 120°C.

HPLC: R_t = 5.4 min
Column: Nucleosil 100 C-18 7µm, 250 x 4 mm

Eluant: methanol/water = 60:40

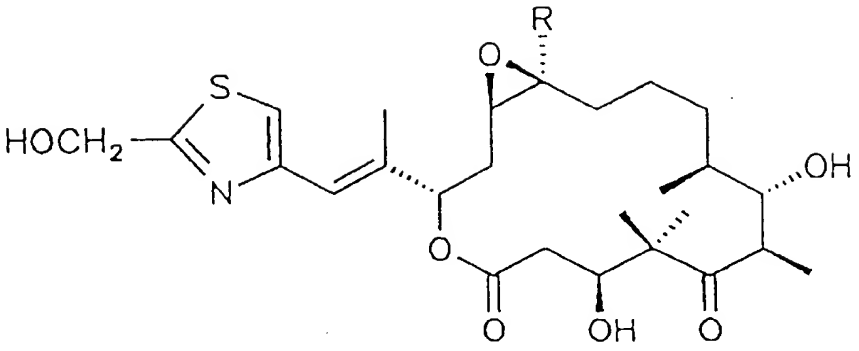
Flow rate: 1.2 ml/min

Detection: diode array

^{1}H-NMR (300 MHz, $CDCl_3$): δ = 2.37 (2-H_a), 2.52 (2-H_b), 4.20 (3-H), 3.27 (6-H), 3.74 (7-H), 1.30-1.70 (8-H, 9-H_2, 10-H_2, 11-H_2), 2.78 (13-H), 1.91 (14-H_a), 2.06 (14-H_b), 5.42 (15-H), 6.58 (17-H), 7.10 (19-H), 4.89 (21-H_2), 1.05 (22-H_3), 1.26 (23-H_3), 1.14 (24-H_3), 0.98 (25-H_3), 1.35 (26-H_3), 2.06 (27-H_3).

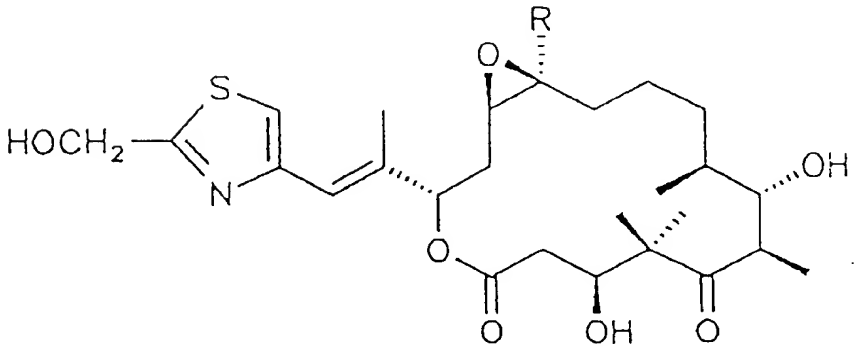
Patent Claims

1.



Epothilon E R = H

2.



Epothilon F R = CH_3

Fig. 1 HPLC analysis of an XAD eluate at the end of fermentation

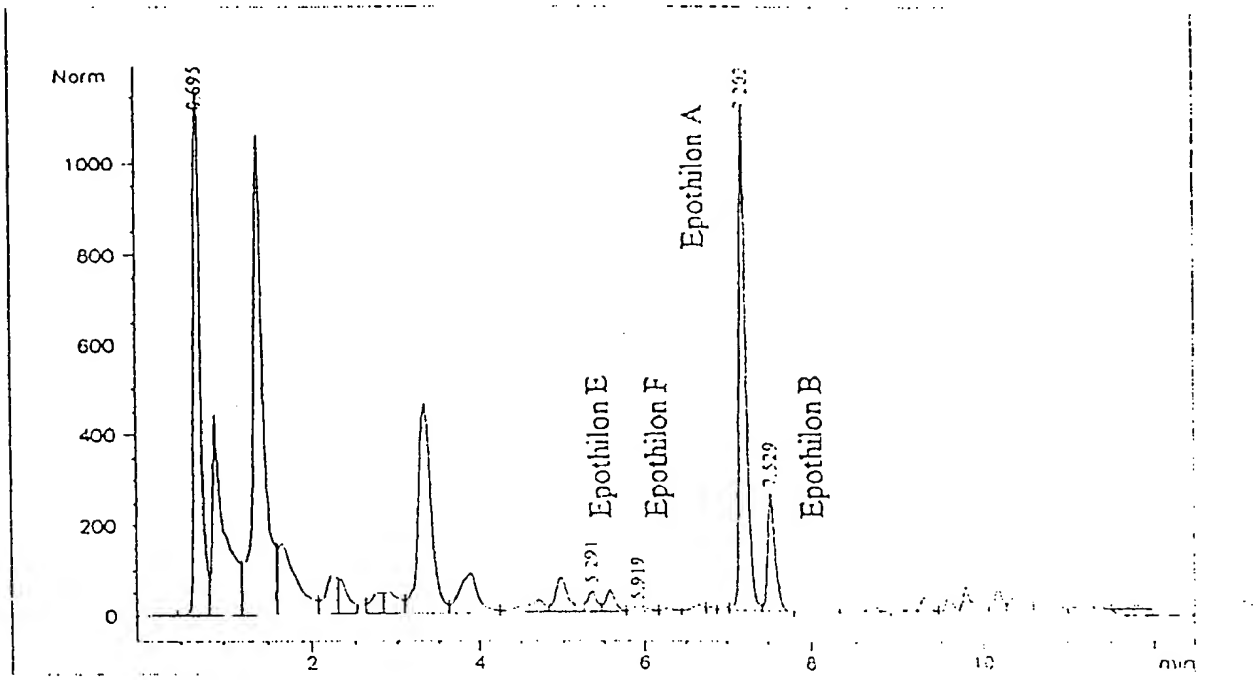


Fig. 2 Concentration of epothilon E and F in a fermentation broth after a mixture of epothilon A and B has been fed in, analysed after 48 hours' incubation

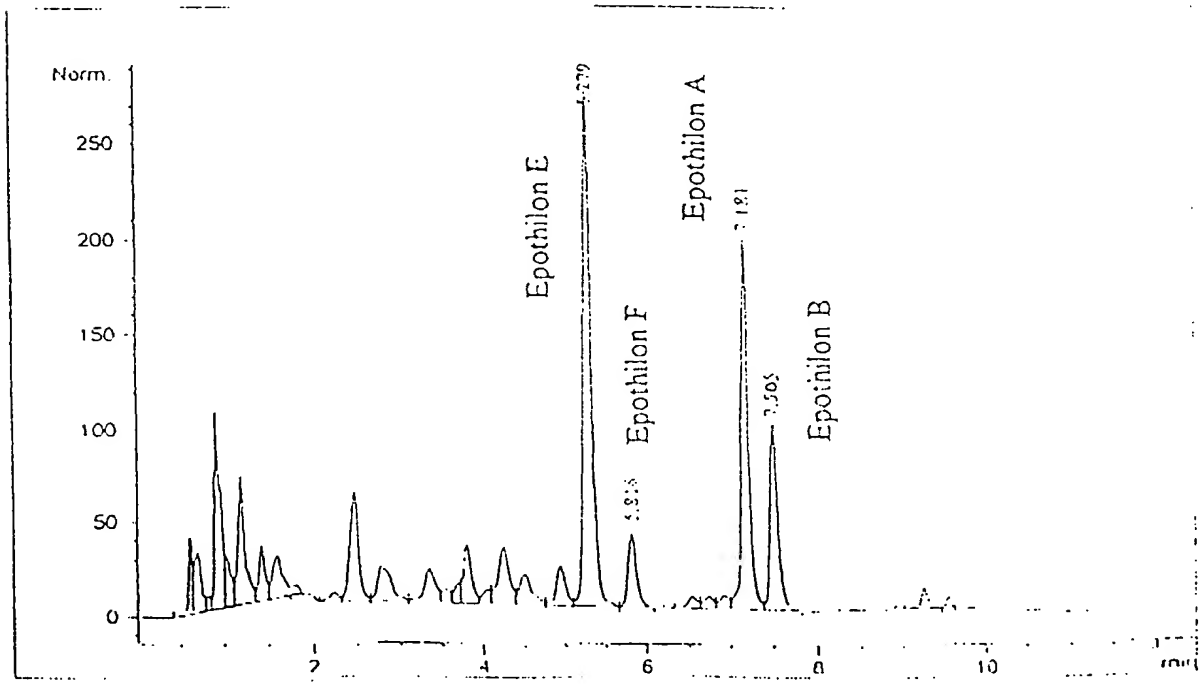


Fig. 3 Kinetics of the biotransformation of epothilon A to epothilon E by *Sorangium cellulosum* So ce90